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Activation of AMP-Activated Protein Kinase Leads to the Phosphorylation of Elongation Factor 2 and an Inhibition of Protein Synthesis

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Summary

Protein synthesis, in particular peptide-chain elongation, consumes cellular energy. Anoxia activates AMP-activated protein kinase (AMPK, see [1]), resulting in the inhibition of biosynthetic pathways to conserve ATP. In anoxic rat hepatocytes or in hepatocytes treated with 5-aminoimidazole-4-carboxamide (AICA) riboside, AMPK was activated and protein synthesis was inhibited. The inhibition of protein synthesis could not be explained by changes in the phosphorylation states of initiation factor 4E binding protein-1 (4E-BP1) or eukaryotic initiation factor 2α (eIF2α). However, the phosphorylation state of eukaryotic elongation factor 2 (eEF2) was increased in anoxic and AICA riboside-treated hepatocytes and in AICA riboside-treated CHO-K1 cells, and eEF2 phosphorylation is known to inhibit its activity. Incubation of CHO-K1 cells with increasing concentrations of 2-deoxyglucose suggested that the mammalian target of the rapamycin (mTOR) signaling pathway did not play a major role in controlling the level of eEF2 phosphorylation in response to mild ATP depletion. In HEK293 cells, transfection of a dominant-negative AMPK construct abolished the oligomycin-induced inhibition of protein synthesis and eEF2 phosphorylation. Lastly, eEF2 kinase, the kinase that phosphorylates eEF2, was activated in anoxic or AICA riboside-treated hepatocytes. Therefore, the activation of eEF2 kinase by AMPK, resulting in the phosphorylation and inactivation of eEF2, provides a novel mechanism for the inhibition of protein synthesis.

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Results and Discussion

Here, we have investigated the involvement of AMPK in the inhibition of protein synthesis associated with stresses linked to changes of the cellular energy status in hepatocytes and cultured cells and whether the phosphorylation of initiation and/or elongation factors could explain this effect. Incubation of hepatocytes under anoxic conditions led to AMPK activation and an inhibition of protein synthesis (Figures 1A and 1B). In order to test the hypothesis that AMPK might be involved in this effect, parallel incubations were conducted in the presence of AICA riboside, a compound that activates AMPK in certain cells following its conversion to AICA ribotide (ZMP), an AMP analog [2, 3]. AICA riboside treatment led to a sustained activation of AMPK, and protein synthesis was markedly inhibited. In anoxia, the activation of AMPK was transient. After 10 min of incubation, AMPK was activated 4.4-fold by anoxia and 3-fold by AICA riboside (Figure 1A), and, during this period, the rates of protein synthesis were decreased by 70% and 40%, respectively (Figure 1B). In hepatocytes treated with AICA riboside, the activation of AMPK and the inhibition of protein synthesis were dose dependent (not shown).

We examined the phosphorylation states of components of the translation initiation steps in anoxic and AICA riboside-treated hepatocytes. No increase in phosphorylation of eIF2α was detected by immunoblotting with an anti-phosphospecific (Ser51) antibody and, indeed, in anoxia, a decrease in phosphorylation was actually observed (see Figure S1 in the Supplementary Material available with this article online). Similarly, no change in the phosphorylation state of 4E-BP1 was observed in anoxic or AICA riboside-treated hepatocytes, and 4E-BP1 was mainly present as the least-phosphorylated α form (see Figure S1 in the Supplementary Material). Since 4E-BP1 is already in this maximum-inhibitory form in control cells, changes in phosphorylation cannot underlie the observed inhibition of protein synthesis.

In contrast, conditions that led to the inhibition of protein synthesis and AMPK activation in hepatocytes increased the phosphorylation state of the elongation factor eEF2, as assessed by immunoblotting of hepatocyte extracts with an anti-phospho (Thr56) eEF2 antibody (Figure 1C). It is noteworthy that eEF2 phosphorylation was higher in anoxic cells than in AICA riboside-treated cells and that, in anoxia, the inhibition of protein synthesis was more pronounced than observed with AICA riboside treatment (Figure 1B). Resolution of the phosphorylated and nonphosphorylated forms of eEF2 by isoelectric focusing indicated that more than 75% of hepatocyte eEF2 was phosphorylated in response to AICA riboside and more than 90% was phosphorylated in anoxia (see

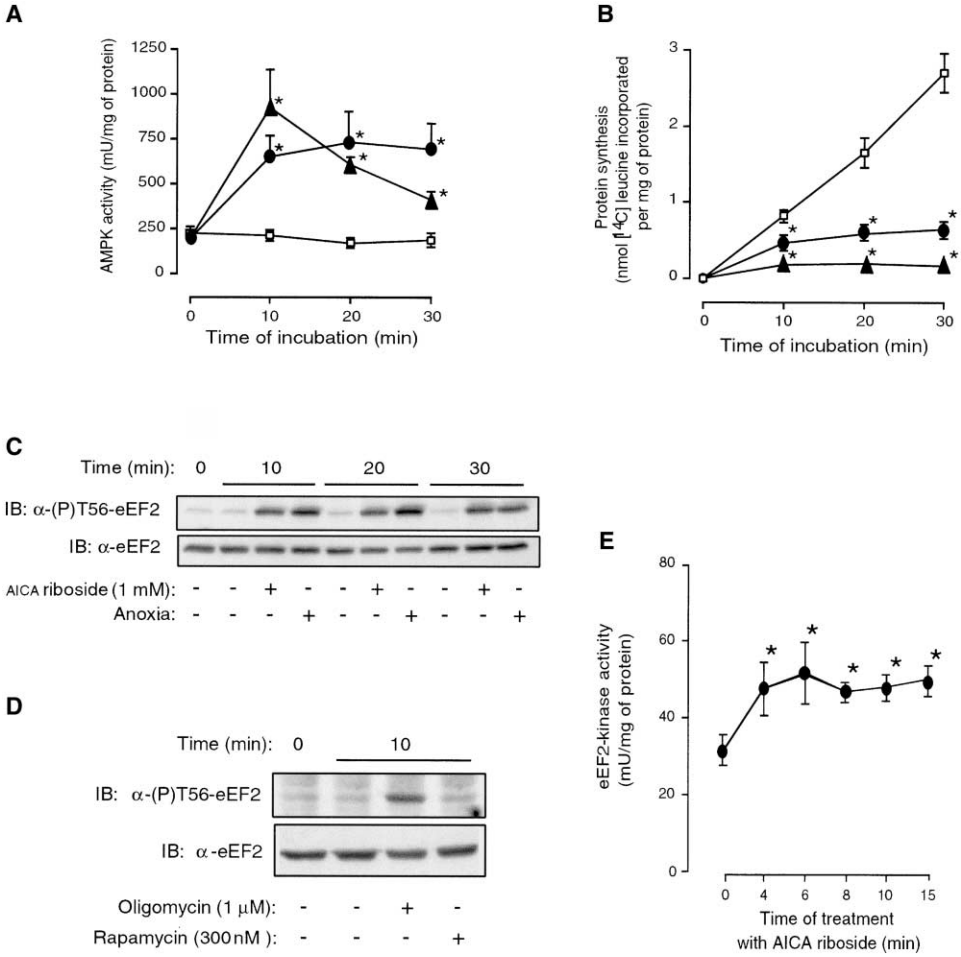

Figure 1. Time Course of the Effects of Anoxia and AICA Riboside on AMPK Activity, Protein Synthesis, eEF2 Phosphorylation, and eEF2 Kinase Activity in Incubations of Hepatocytes

(A–E) Hepatocytes were incubated under normoxic conditions (open squares), under anoxia (filled triangles), or in the presence of 1 mM AICA riboside (filled circles). At the indicated times, cells were collected by centrifugation for (A) AMPK assay or for the (B) measurement of protein synthesis, as described in the Experimental Procedures. The results are the means ± SEM of at least three separate experiments. In (C), extracts (25 μg) were subjected to SDS-PAGE and were immunoblotted with anti-phospho (Thr56) eEF2 antibody (upper panel). The membrane was stripped and probed with an anti-full-length eEF2 antibody that does not distinguish the phosphorylated protein (lower blot). In (D), hepatocytes were preincubated with and without rapamycin and were incubated in the presence and absence of oliogomycin for 10 min. Extracts were then immunoblotted as in (C). In parallel incubations, it was verified that rapamycin was effective, as the glutamine-induced activation of p70S6K was blocked (not shown). For (C) and (D), the results of a representative experiment are shown. In (E), hepatocytes were incubated with 1 mM AICA riboside, and extracts were prepared at the indicated times for eEF2 kinase assay with 1 μM free Ca^{2+}. The results are the means ± SEM of three separate experiments. An asterisk indicates a significant activation of eEF2 kinase ($p < 0.05$) compared with the control.

Figure S2 in the Supplementary Material). In CHO-K1 cells treated with AICA riboside, the increase in eEF2 phosphorylation was maximal after 20–30 min of incubation and was dependent on the dose of AICA riboside (see Figure S3 in the Supplementary Material). In these cells, eEF2 phosphorylation also increased following treatment with the uncoupler carbonyl cyanide m-chlorophenyl-hydrazone (not shown).

mTOR has been suggested to act as an ATP sensor with a K_m for ATP for the phosphorylation of p70 ribosomal protein S6 kinase (p70S6K) and 4E-BP1 of about 1 mM [4]. Moreover, p70S6K lies downstream of mTOR and phosphorylates and inactivates eEF2 kinase, the effect being blocked by rapamycin [5]. Therefore, we investigated the possible involvement of the inhibition of mTOR signaling in the increased phosphorylation of eEF2 in CHO-K1 cells incubated with increasing concentrations of 2-deoxyglucose to progressively deplete intracellular ATP. In these experiments, we verified that AMPK was activated by monitoring the increase in phosphorylation of Ser79 of acetyl-CoA carboxylase (Figure 2). With increasing 2-deoxyglucose concentrations, leading to a maximum level of eEF2 phosphorylation, there was little reduction in the already substantial extent of phosphorylation of p70S6K and 4E-BP1, as assessed by band shifts, and the phosphorylation state of ribosomal protein S6 was unaffected (Figure 2). Therefore, a reduction in mTOR signaling cannot explain the

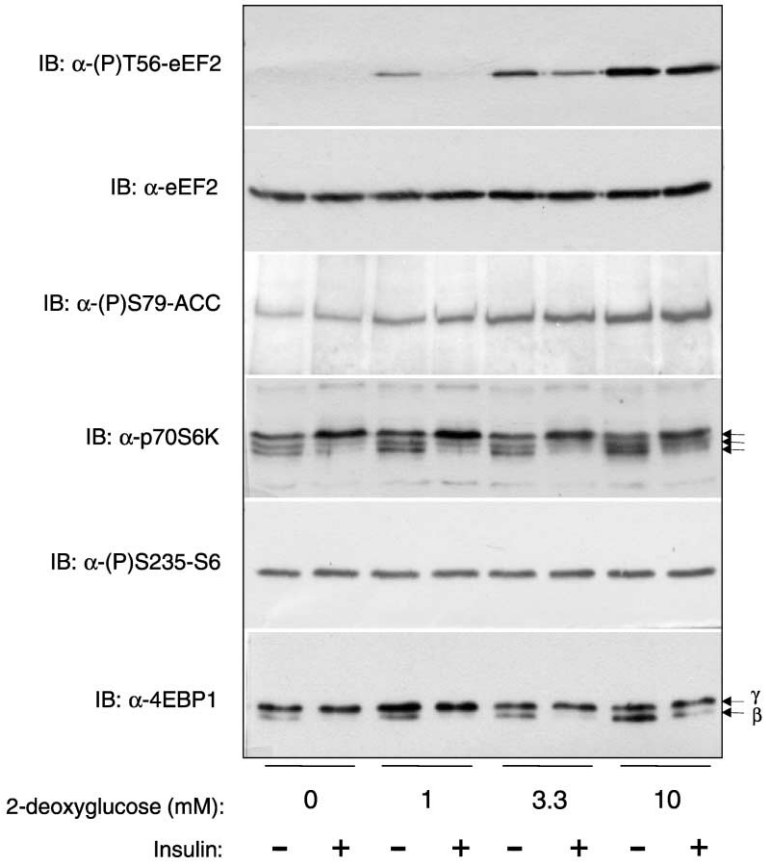

Figure 2. Effect of Increasing Concentrations of 2-Deoxyglucose on the Phosphorylation State of eEF2 and Downstream Components in the mTOR Signaling Pathway

Serum-fed CHO-K1 cells were incubated with the indicated concentrations of 2-deoxyglucose in the presence and absence of insulin (0.1 μM) Extracts were prepared as described in the Experimental Procedures and were blotted with the antibodies indicated following SDS-PAGE. α-(P)S79-ACC was used to monitor AMPK activation corresponding to the phosphorylation site for AMPK in acetyl-CoA carboxylase (Ser79). For 4E-BP1, two bands are visible, the lower β and upper γ forms. For p70S6K, the three bands indicated by arrows represent different phosphorylated species, and insulin causes a shift to the slowest most-phosphorylated form.

increase in eEF2 phosphorylation in cells incubated with low 2-deoxyglucose concentrations to activate AMPK. At 10 mM 2-deoxyglucose, there was a hint of a band shift for p70S6K, which would indicate a decrease in activity. Thus, at very high 2-deoxyglucose concentrations (Dennis et al. used up to 100 mM 2-deoxyglucose in their studies, [4]), which severely deplete ATP levels [4], a decrease in p70S6K via a decrease in mTOR signaling could contribute to eEF2 phosphorylation via an increase in eEF2 kinase activity.

In isolated hepatocytes, both the basal and anoxia-induced inhibition of protein synthesis were unaffected by rapamycin (not shown). Furthermore, rapamycin had no effect on the basal (Figure 1D) and anoxia-induced phosphorylation of eEF2 (not shown), and this suggests that p70S6K is not active and that mTOR is not mediating these effects. For AMPK to inhibit protein synthesis by interfering with mTOR-dependent signaling, the system would first need to be activated, e.g., by amino acids or insulin. In isolated hepatocytes, p70S6K activity is very low under basal conditions, but it can be activated upon incubation with amino acids, via mTOR, and this activation is blocked by rapamycin [6]. Indeed, the amino acid-dependent phosphorylation and activation of p70S6K in hepatocytes was recently shown to be inhibited by AMPK activation [7, 8]. Also, AMPK activation by AICA riboside in rat skeletal muscle in vivo inhibited both the mTOR pathway and protein synthesis [9].

HEK293 cells were transfected with dominant-negative α1 AMPK to provide additional evidence that AMPK activation inhibits protein synthesis. AMPK is not activated by AICA riboside treatment in these cells [10]. However, as demonstrated previously [10], AMPK can be activated in HEK293 cells by treatment with oligomycin (Figure 3A). Transfection with the dominant-negative α1 AMPK construct did not decrease basal AMPK activity, as shown previously [10]. This might be due to the trimeric nature of AMPK and the existence of both α1 and α2 catalytic subunits. However, transfection with a dominant-negative α1 AMPK mutant almost completely abolished AMPK activation by oligomycin (Figure 3A). In our previous studies conducted under exactly the same conditions, there was no effect of transfection of the dominant-negative construct on the levels of endogenous or transfected AMPK isoforms, as verified by immunoblotting [10]. A wild-type α1 AMPK vector was used as a control and did not increase AMPK activity, as demonstrated previously [10]. Transfection with dominant-negative α1 AMPK slightly decreased the basal rate of protein synthesis, but the effect was not statistically significant (control rates of protein synthesis were 0.15, 0.15, and 0.17 nmol/min/mg of protein and 0.12, 0.12, and 0.15 nmol/min/mg in the dominant-negative α1 AMPK-transfected cells). In cells treated with oligomycin, the rate of protein synthesis was decreased by about 60%, and transfection with the dominant-negative α1 AMPK blocked its inhibitory effect (Figure 3B). Moreover, transfection of dominant-negative α1 AMPK reduced the oligomycin-induced phosphorylation of eEF2 to control levels (Figure 3C).

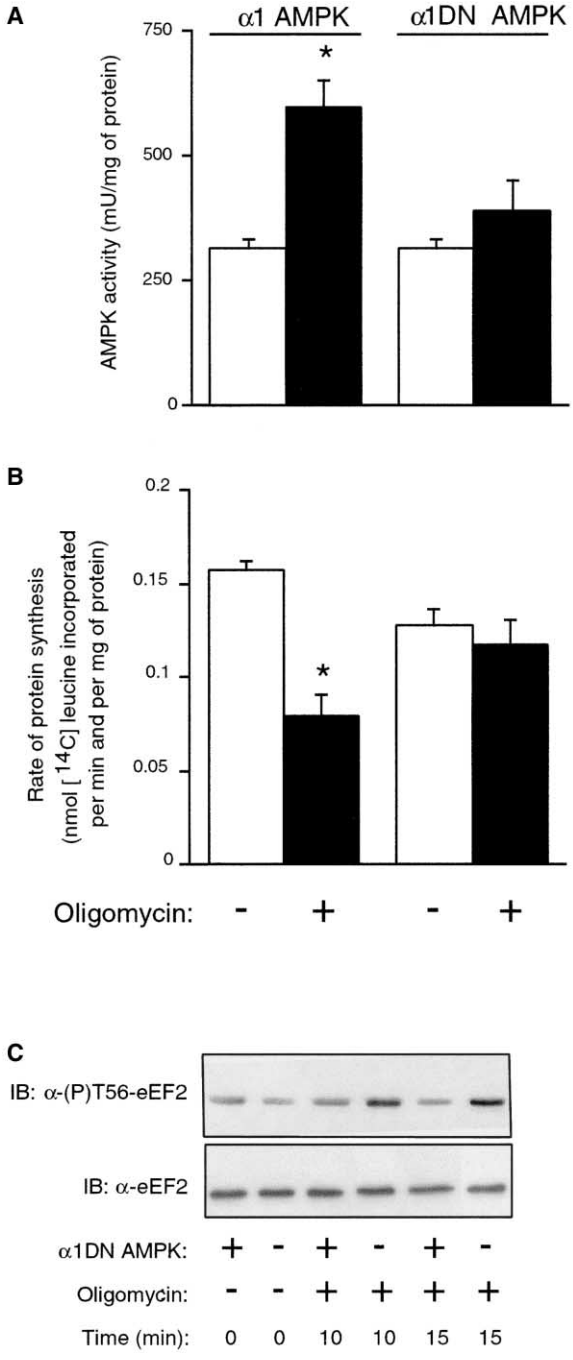

Figure 3. Effect of Dominant-Negative AMPK on the Oligomycin-Induced Activation of AMPK, Protein Synthesis Inhibition, and eEF2 Phosphorylation in Transfected HEK293 Cells

(A–C) HEK293 cells were transfected with 10 μg α1 AMPK DNA or 10 μg α1DN AMPK DNA (see the Supplementary Material). Cells were incubated with and without oligomycin for 15 min for the measurements of (A) AMPK activity and (B) protein synthesis. The results are the means ± SEM of three separate experiments. An asterisk indicates a significant effect of oligomycin ($p < 0.05$) compared with the controls. In (C), extracts were blotted with anti-phospho (Thr56) eEF2 antibody or anti-full-length eEF2 as a loading control, and results of the time course of a typical experiment are shown.

We tested whether eEF2 was a direct substrate for AMPK. Although the protein could be phosphorylated by AMPK in vitro, the stoichiometry of phosphate incorporation was low, reaching a plateau of 0.1–0.2 mol/mol after 30 min of incubation, indicating that eEF2 is a poor substrate (not shown). This low stoichiometry of phosphorylation could not be increased by prior dephosphorylation of eEF2 with protein phosphatase 2A catalytic subunits, and the anti-eEF2 Thr56(P) antibody revealed that AMPK did not phosphorylate this residue (not shown). In addition, the sequence around Thr56 of eEF2 does not fit the consensus requirements for AMPK [1]. We therefore tested whether eEF2 kinase itself was activated by conditions that activated AMPK. In hepatocytes incubated for 10 min in anoxia (not shown) or with AICA riboside (Figure 1E), eEF2 kinase was activated by about 2-fold. The activation was rapid, reaching a maximum after 6 min of treatment with AICA riboside (Figure 1E).

The inhibition of protein synthesis by oxygen deprivation is a well-known observation [11–16]. Surprisingly, the molecular mechanisms behind this phenomenon have received little attention, although changes in the association of 4E-BP1 and eIF4E have been reported [17, 18]. Here, we confirm that anoxia inhibits protein synthesis in isolated hepatocytes and, under these conditions, AMPK was activated and the phosphorylation state of eEF2 was increased. Likewise, AICA riboside inhibited protein synthesis and increased eEF2 phosphorylation. The phosphorylation and inactivation of eEF2 is a logical mechanism for pausing translation temporarily at elongation. Translation could then resume quickly following a return to normal ATP levels. Regulating initiation, on the other hand, would cause polysomes to dissociate and would have varying effects on different mRNAs. In this respect, it is noteworthy that, in hepatocytes, the shut down in protein synthesis after 30 min of anoxia can be fully reversed upon reoxygenation [11].

Purified rat liver eEF2 was a poor substrate of AMPK, which suggests that the increase in eEF2 phosphorylation might be a consequence of eEF2 kinase activation rather than direct phosphorylation of eEF2 by AMPK. Indeed, eEF2 kinase was activated by anoxia or AICA riboside treatment, suggesting that AMPK activation mediates this effect.

Supplementary Material

Supplementary material including the Experimental Procedures can be found at http://images.cellpress.com/supmat/supmatin.htm. Also included are figures showing the effects of various treatments on the level of Ser51 phosphorylation of eIF2α in hepatocytes and CHO-K1 cells together with the effects of treatments on the phosphorylation state of 4E-BP1 in hepatocytes (Figure S1). Other figures show isoelectric focusing and eEF2 detection in extracts from anoxic and AICA riboside-treated hepatocytes (Figure S2) and a time course and dose dependence of eEF2 phosphorylation in CHO-K1 cells (Figure S3).

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